Evaluation of SD-208, a TGF-β-RI Kinase Inhibitor, as an Anticancer Agent in Retinoblastoma

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Abstract- Retinoblastoma is the most common intraocular tumor in children resulting from genetic alterations and transformation of mature retinal cells. The objective of this study was to investigate the effects of SD-208, TGF-β-RI kinase inhibitor, on the expression of some miRNAs including a miR-17/92 cluster in retinoblastoma cells. Prior to initiate this work, the cell proliferation was studied by Methyl Thiazolyl Tetrazolium (MTT) and bromo-2′-deoxyuridine (BrdU) assays. Then, the expression patterns of four miRNAs (18a, 20a, 22, and 34a) were investigated in the treated SD-208 (0.0, 1, 2 and 3 µM) and untreated Y-79 cells. A remarkable inhibition of the cell proliferation was found in Y-79 cells treated with SD-208 versus untreated cells. Also, the expression changes were observed in miRNAs 18a, 20a, 22 and 34a in response to SD-208 treatment ($P<0.05$). The findings of the present study suggest that the anti-cancer effect of SD-208 may be exerted due to the regulation of specific miRNAs, at least in this particular retinoblastoma cell line. To the best of the researchers' knowledge, this is the first report demonstrating that the SD-208 could alter the expression of tumor suppressive miRNAs as well as oncomiRs *in vitro*. In conclusion, the present data suggest that SD-208 could be an alternative agent in retinoblastoma treatment.

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Keywords: miRNA; Retinoblastoma; SD-208; Y-79 cell line

Introduction

Retinoblastoma (Rb), the most common pediatric cancer of the eye, exists in sporadic and heritable forms (1). Two kinds of this cancer, bilateral (15% inheritable) and unilateral (85% non-heritable) have been reported. This malignancy occurs as part of a familial cancer syndrome; however, the main ratio of retinoblastoma arises with no preceding family history (2). Retinoblastoma is first cancer confirmed to have a genetic origin (2,3). Although genetic changes in Rb gene have been frequently reported in retinoblastoma, the genetic alteration in other biomolecules and genes such as MYCN oncogene appears to be important in its pathogenesis (4-8). Studies have shown that inappropriate expression or loss of RB function is associated with other human cancers (9-13).

Today, it is well known that numerous biomolecules such as micro RNAs (miRNAs) and long non-coding RNAs (lncRNAs) are implicated in the regulation of various human cancer genes (14-19). For instance, these molecules play a vital role in tumor suppressor networks such as RB gene (20). The expression profile and biological significance of miRNAs depend on the target genes and kind of malignancy (tumor microenvironment). Many researchers determined that a specific group of miRNAs is highly expressed in multiple tumor types and turn on some oncogenes while overexpression of another group leads to the activation of some tumor suppressor genes. It is quite

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understandable, therefore, that these molecules could be used as biomarkers and targets for the cancer diagnosis and therapy, respectively.

In spite of the fact that the survival rate of retinoblastoma is about 98%, chemotherapeutic resistance and various side effects of current therapies are considered the main challenges for this disease. Studies have shown that the pretreatment evaluation of retinoblastoma cases is individualized and directed by the chosen mode of therapy. The present study, for the first time, investigated the effect of a TGβRI kinase inhibitor, SD-208, on the regulation of four miRNAs 18a, 20a, 22 and 34a in retinoblastoma

Materials and Methods

Cell culture

The human retinoblastoma Y-79 cell line was obtained from National Cell Bank of Iran (NCBI) affiliated to Pasteur Institute (Tehran, Iran). The cells were grown in suspension at a concentration of 105-106 cells/ml in RPMI-1640 medium (Gibco; Germany) containing 10% fetal bovine serum (FBS) (Gibco; Germany), 2 mM L-glutamine, 1 mM sodium pyruvate, and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin). Cell culture condition designed at 37°C in a humidified atmosphere of 5% CO2-95% air.

SD-208 treatment

The treatment of cells with SD-208 (Sigma-Aldrich, Belgium) was conducted as previously described (21). Briefly, SD-208 was dissolved in dimethyl sulfoxide (DMSO) with a concentration of 20 μM and stored at -20°C. The Y-79 cells in each experiment were treated using a different range of SD-208 concentrations (as explain in the below sections).

MTT assay

The cytotoxicity effect of SD-208 was measured by the MTT assay as previously described (21,22). Briefly, Y-79 cells were seeded in 96-well flat-bottom microtitration plates (SPL Life Sciences; South Korea) at a density of 4×104 cells/well (200 μl media/well). After reaching to ~85% confluency, the cells were treated with various concentrations of SD-208 (0.0, 1, 2, 3 μM). In all in vitro experiments, control cells were incubated with dimethyl sulfoxide (DMSO) alone (with a final concentration 0.2%).

BrdU assay

The BrdU assay was performed using BrdU ELISA kit as previously described (21). Briefly, the Y-79 cells were seeded in 96-well flat-bottom microtitration plates at a density of 4×104 cells/well (100 μl media/well). Then, the cells were treated with SD-208 (0.0, 1, 2, 3 μM) and the cell apoptosis calculated as previously described (21).

Total RNA extraction

The isolation of total RNA from cells with a cell density of 80% confluent was conducted using Trizol® total RNA isolation reagent (Gibco BRL, Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. To remove any genomic contamination, the total RNA was treated with RNase-free DNase I and stored at -70° C for further tests.

miRNA expression analysis by Real-time q-PCR

Four miRNAs (including miR-18a, 20a, 22, 34a) genes involving in retinoblastoma were selected from the Sanger Center miRNA Registry at http://www.sanger.ac.uk/Software/Rfam/mirna/index.shtml.

The microRNAs expression was analyzed by real-time quantitative polymerase chain reactions (qPCR) using the SYBR Green method by the specific primers for mature forms of aforementioned micro RNAs as per the manufacturer's instructions (Parsgenom, Iran). The real-time q-PCR carried out by poly (A) tailing method relying on polyadenylation, followed by cDNA synthesis using a universal oligo(dT) primer. RNU6B was used as an endogenous (internal) control, and the data were normalized to the expression level of this housekeeping gene.

Our protocol consisted of two steps: reverse transcription (RT) and real-time PCR. First, RNA was transcripted to complementary DNA (cDNA) using the poly-adenylation procedure. Briefly, a 20 μl reaction including 2 μg total RNA, 2 μl of 10X reaction buffer, 2 μl of 10 mM ATP and 1 unit of Poly (A) polymerase was incubated at 38 °C for 30 min, followed by enzyme inactivation at 65 °C for 3 min. After polyadenylation, the cDNA was synthesized using 2 μg of polyadenylated RNA (RNA poly A tail) and miR-cDNA Syn specific primers in a reverse transcription reaction. This reaction was performed in a volume of 10 μl containing 1 μl of the polyadenylation reaction product, 1 μl of 0.5 mM universal RT primer, 1 μl of 10 mMdNTP, 0.5 μl of RT enzyme in. The reaction was incubated at 42 °C for 60 min and then terminated by heating at 85 °C for 5 min.

Diluted cDNAs then were amplified in a 20μl reaction containing SYBER Green Mastermix, forward

and reverse miR-specific primers (each 1 µl) and DEPC-treated distilled water by 35 cycles of PCR amplification under the following conditions: denaturing at 95 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. Real-time PCR was performed on a Bio-Rad CFX96 Real-Time PCR System.

All the reactions were performed in triplicate. Specificity of primers was verified by observing a single peak dissociation curve for each run. The threshold cycle (CT) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. CT values were converted into total copy numbers using a standard curve. The absence of contamination was verified using non-template controls.

Statistical analysis

Statistical analysis for multiple comparisons of differences between treatment groups was performed with SPSS software. The standard error of means was computed, and analysis of variance (ANOVA, Tukey post tests) completed via GraphPad Prism 5.0 software. Gene expression analysis was performed by $2^{-\Delta\Delta ct}$ method via GraphPad Prism 5.0 software. *P*<0.05 were considered as statistically significant.

Results

SD-208 decreases proliferation of and induces apoptosis in Y-79 cells

To determine the inhibitory effect of SD-208on cell proliferation in Y-79 cells, the cell growth was analyzed by performing MTT assay with various conditions. The data resulted from the present study clearly showed that SD-208treatment significantly reduces the viability of Y-79 cells in a dose- and time-dependent manner. Figure1 shows the cell response to different drug concentrations (0.0, 1, 2, 3 µM) for a time course of 24, 48, and 72 h. The results of cell function assays revealed a significant change in the cell viability in the treated versus untreated Y-79 cells (*P*<0.05), especially at 48h and 72h.

Moreover, to examine whether SD-208can promote apoptosis in Y-79 cells, we analyzed apoptotic index using a BrdU experiment in the SD-208treated cells compared to controls (Figure 2). The apoptotic findings revealed a significant change in cell death in the treated versus control cells (*P*<0.05) at 48h and 72h.

The obtained results show that the cytotoxicity and apoptosis effects of SD-208on retinoblastoma cell line are time and dose-dependent. Besides, its apoptotic effect like its toxicity seems to follow a time and dose-dependent manner.

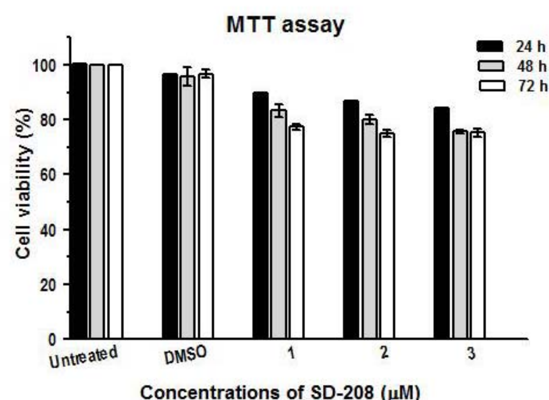

Figure 1. Effect of SD-208 on the Y-79 cell viability. Y-79 cells were treated by 0.0, 1, 2 and 3 µM for different time courses. The cell toxicity was examined by MTT assay and data was reported as the percentage change in comparison with the controls. Comparison of the cell viability of Y-79 cells in different concentrations of SD-208 to controls was analyzed by one-way ANOVA test. Results were expressed as mean ±SEM from three independent experiments. *P*<0.05 was considered as statistically significant

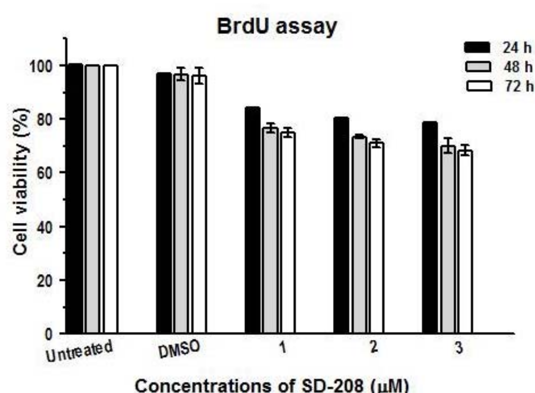

Figure 2. Impact of SD-208 on the Y-79 cell proliferation. The Y-79 cells were treated by 0.0, 1, 2 and 3 µM for different time courses. Cell apoptosis was examined by BrdU assay and analysis of one-way ANOVA was used to compare the cell apoptosis. Results were expressed as mean ±SEM from three independent experiments. *P*<0.05 was considered as statistically significant

SD-208regulates miRNA expression in retinoblastoma

To study the ability of the SD-208as a potential anticancer agent via changing oncogene/tumor suppressor miRNAs, the researchers partially evaluated its effects on some miRNA expression. As Figure 3

shows, SD-208could significantly upregulate miR-18a, 22a, and 34a (*P*<0.05) while downregulating miR-20a (*P*<0.05) expression in retinoblastoma cells.

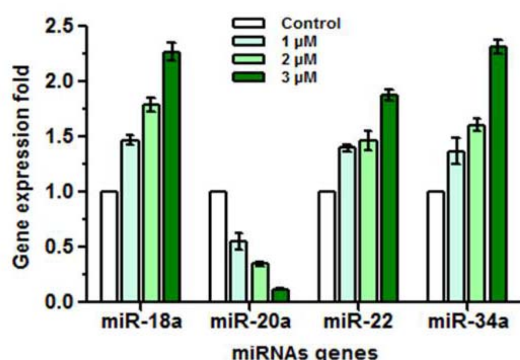

Figure 3. Effect of SD-208 on miRNA expression. Evaluation of the ffects of SD-208 on miRNA expression was completed by Real-time q-PCR. SD-208 could significantly upregulate miR-18a, 22a, and 34a (*P*<0.05) while downregulating miR-20a (*P*<0.05) expression

Discussion

It is well established that TGFβ signaling pathway plays a critical role in the normal and abnormal cellular processes. In this study, we focused on the involvement of miRNAs in the anticancerous effect of SD-208. We found that the kinase inhibitor, SD-208, was able to inhibit TGF-β-evoked proliferation in retinoblastoma via regulation of cancer-related miRNAs. A few reports suggested that SD-208 could not be an effective chemotherapeutic agent in at least some human cancers (21,23). Although in a study it was shown that SD-208 could decrease the tumor angiogenesis in athymic nude mice, it was not able to inhibit mammary tumor growth and metastasis. Most recently, Akbari *et al*., reported no significant reduction in cell proliferation and angiogenesis in human colon adenocarcinoma cells treated with SD-208 (21).

In contrast, to work described above, accumulated data have demonstrated the potential anti-cancer activities of SD-208 (24-26) in distinct malignancies such as pancreatic (27,28), glioma (25) and melanoma (24). Mohammad KS defined SD-208 as promising novel agents for the treatment of human malignant glioma and other conditions associated with pathological TGFβ activity. Also, Uhl M demonstrated that the targeting of TGF-β signaling pathway by SD-208 may prevent the development of melanoma bone metastases. However, these reports do not address exactly how SD-208 regulates miRNA expression and the genes which are implicated in the cancer therapy.

Our study identified that miR18a to be modulated by SD-208. The miR18a overexpression seems to be an important factor in cancer treatment. Krutilina *et al.* reported that miR18a expression suppressed distant metastasis via the hypoxia-inducible factor 1-alpha pathway (29). Interestingly, it is well known that neglected or untreated retinoblastoma cases can demonstrate extraocular spread, primarily through the optic nerve (30,31). Based on these observations, it might be possible that the overexpression of miR18a could be implicated in repressing the metastatic behavior of intraocular retinoblastoma. In addition, miR-34 and miR-22 in our study following real time RT-PCR validation demonstrated a significant upregulation in treated Y-79 with SD-208 cells as compared to the controls. The miR-34 family has three members, miR-34 a,b,c. It has been thought that miR-34a is an important regulator of tumor suppression. This molecule controls different aspects of cell cycle processes such as differentiation and apoptosis; Its differential expression has been detected in different cancer such as breast, melanoma, pancreatic, and prostate cancer cell lines (32). However, the exact molecular mechanisms by which it acts as an anti-oncogenic activity is not well known. Various studies demonstrated that miR-34a is regulated by diverse approaches such as p53-dependent (33-35) and p53-independent mechanisms (36-38). TGFβ signaling pathway as a molecular target for the miR-34a could also be another proposed model of function. More recently, a study suggested the capability of miR-34a to suppress epithelial-mesenchymal transition (EMT) in human cancer through TGFβ signaling pathway (39). In addition, a cross talk between miR-34a, as a complex network interaction could interpret the miR-34a-mediated apoptosis in cancer. In this regard, a study using Y79 cells demonstrated that the expression of miR-34 was elevated after p53 activation (40). Our results, consistent with previous studies, recommend that miR-34a-triggered cell apoptosis in response to the SD-208 might open a new window for better understanding and application of the kinase inhibitor-based therapy.

Moreover, miR-20a was significantly downregulated in treated retinoblastoma cells with SD-208 compared to controls in this study. miR-20 belongs to the miR-17/92 cluster and acts as an important oncogene in several cancers such as retinoblastoma (41) and targets genes important in regulating cell proliferation and cancer development. Previous studies have reported that this miRNA is involved in cellular proliferation (42) cell

cycle (43) and invasion activity (41). As it mentioned, Y-79 cells do not functionally express TGFβRI, a key component for TGFβ signal transduction. Altogether, these observations postulate another alternative system by which miR-20a exerts its normal and abnormal biological function.

The miR-17-92 cluster comprises six miRNAs: miR-17, miR-18a, miR-19a, miR-19b, miR-20a , and miR-92a (44). It has been well characterized that the miR-20a acts as an oncogene in various malignancies cells by targeting different cell signaling pathways (44-48). Evidence that the miRNA exhibits oncogenic effects in retinoblastoma came from distinct studies (49-53). Our results in agreement with these studies approved the miR-20a could potentially act as an oncogene in retinoblastoma.

The most obvious reason to account for the mechanism(s) by which SD-208 could regulate the miRNAs in retinoblastoma could be due to different scenarios. Studies have reported the absence of TGFβRI in some cancerous cells such as Y-79 (54) and SW48 cells (55). As mentioned before, SD-208 can block the biological effects of TGFβ cascades by inhibiting of the kinase activity of TGFβRI. In the present study also, we analyzed the TGFβRI and TGFβRII mRNA expression in the cells treated with and without the SD-208. We could not detect their mRNA in all tested samples (data not shown). Considering this phenomenon; it would be useful to explain an appropriate relationship between SD-208 and miRNA regulation under this circumstance. Our previous results showed that SD-208 could regulate miRNA-135b in colorectal cancer (55). Taken together, our findings support the notion that SD-208 could potentially regulate the miRNA expression by indirectly affecting the TGFβ signaling (also we called a non-canonical pathway).

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